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### Authors
Jones, AG
Stockwell, CA
Walker, D
et al.



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# The Molecular Basis of a Microsatellite Null Allele From the White Sands Pupfish

**A. G. Jones, C. A. Stockwell, D. Walker, and J. C. Avise**

Microsatellite loci were cloned and characterized from the White Sands pupfish (*Cyprinodon tularosa*), a New Mexico state-listed endangered species. One locus exhibited a high-frequency nonamplifying allele localized to a single population. This null allele was PCR amplified by redesign of one of the original primers and multiple individuals homozygous for null as well as for nonnull alleles were sequenced using the new primer. These molecular dissections revealed that the original failure to amplify some alleles from this microsatellite locus was due to a 4 bp deletion in one of the original PCR priming sites. Furthermore, the reamplifications revealed five distinct size classes of alleles that had been masquerading as the original null. These null alleles did not overlap in length with the nonnull alleles, and they also differed consistently by a linked nucleotide substitution. Results suggest that the original null allele (as well as the nonnull class) has diversified considerably since its origin and has not recombined frequently with the nonnull class of alleles.

Microsatellite markers have become valuable tools for studies involving population genetics, kinship, and parentage assessment (Bruford and Wayne 1993; Queller et al. 1993), but a potential complication arises from the presence of nonamplifying or null alleles (Pemberton et al. 1995). Null alleles can produce serious problems for population-level studies by creating an apparent excess of homozygotes, resulting in incorrect allele frequency estimates and overestimates of inbreeding. In parentage studies they can result in false exclusions. Null alleles have been encountered in studies of numerous organisms, including mammals (Hulme et al. 1994; Phillips et al. 1993), birds (Primmer et al. 1995), fish (Jones and Avise 1997), insects (Cooper et al. 1996; Oldroyd et al. 1996), and crustaceans (Tam and Kornfield 1996). Despite the pervasiveness of microsatellite null alleles and the difficulties associated with their assay, little is known about their molecular basis (but see below), and even less is known about the evolutionary histories of the null alleles relative to their nonnull allele counterparts.

The state-listed endangered White Sands pupfish (*Cyprinodon tularosa*) is known from only four locations (Salt Creek, Lost River, Malpais Spring, and Mound Spring), all of which are in New Mexico. During the cloning and characterization of microsatellites from this species we encountered a null allele at high frequency in the Malpais Spring population. Here we investigate the molecular basis of this null allele and assess its evolutionary relationships to the nonnull alleles by redesigning PCR primers and sequencing multiple alleles at the region surrounding the microsatellite locus.

## Materials and Methods

### Microsatellite Cloning

Total genomic DNA was extracted from a single *C. tularosa* specimen (from the Salt

Creek population) by a standard phenol/chloroform procedure. The DNA was digested with *Nde*II and size selected by excising the 300–700 bp fragments from a 2% agarose gel after electrophoresis. Fragments were purified using the Prep-A-Gene DNA Purification System (BioRad) and ligated into *Bam*HI-digested, dephosphorylated pBluescript phagemid (Stratagene). Ligations were heat-shock transformed into competent XL1-Blue *E. coli* (Stratagene). The transformation was spread on LB plates containing ampicillin and X-gal. Approximately 1000 white colonies were picked to four patch plates for screening. Colonies were transferred to Hybond-N nylon membranes (Amersham International) following the manufacturer's recommendations and probed twice at 42°C (in 6× SSC, 0.1% SDS, 5× Denhardt's reagent) with two different cocktails of end-labeled oligonucleotides. Each hybridization was followed by two 30 min washes at 42°C in 6× SSC, 0.1% SDS. First, the oligonucleotides $(GT)_{10}$, $(GGAT)_4$, $(GACA)_4$, and $(TAG)_6$ were used, followed by stripping and reprobing with $(GA)_{10}$, $(GATA)_4$, $(TTAGGG)_3$, and $(TTC)_5$. Phagemid DNA was prepared from the 12 colonies that hybridized to the probes (Qiagen QIAprep spin plasmid miniprep kit). The inserts were sequenced using the *fmol* DNA sequencing system (Promega) and primers were designed for microsatellite-containing loci.

The PCR was performed in 10 μl reaction volumes containing 1× Promega *Taq* buffer, 1.5 mM $MgCl_2$, 0.15 μM of each primer, 0.1 mM of each dNTP, and 0.5 units of Promega *Taq* polymerase. The thermal cycling consisted of 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, preceded by 2 min of denaturation at 94°C and followed by an additional 4 min extension at 72°C. Microsatellite polymorphisms were detected by labeling one primer with 1 μCi $\gamma$-$^{32}$P ATP per 5 pmol of primer and electrophoretically resolving fragments on standard 6% polyacrylamide denaturing sequencing gels.

### Null Allele Analysis

A high-frequency null allele was suspected for locus WSP11 in the Malpais Spring population of pupfish because many individuals failed to amplify from the original PCR primer pair (WSP11UP, 5′-AACAAATCCAATAATGTATTAGAA-3′; and WSP11LO, 5′-GATGAACGAGGAGAAAGAATAG-3′), despite the fact that they amplified successfully for other loci. To solve this problem a new PCR primer (WSP11LO-A; 5′-CCCCTGCTGCCTCAAAG-3′) external to WSP11LO was designed from the original cloned sequence. When used in conjunction with WSP11UP, the new flanking primer (WSP11LO-A) produced excellent amplification of the WSP11 locus from all assayed individuals from all four populations.

To determine the molecular basis of the original null allele, several individuals ($N = 5$) that yielded no PCR product from the original primers, and that were homozygous when assayed with the new primer set (WSP11UP and WSP11LO-A), were sequenced using the *fmol* kit (Promega). We also sequenced 10 individuals that were recorded as homozygotes with both the original and new primer pairs, and thus did not contain the null allele.

## Results

### The Null Alleles

Amplification of a sample of 30 individuals from the Malpais Spring population with the original WSP11 PCR primers resulted in eight apparent homozygotes for a fragment 177 bp in length. The other 22 individuals yielded no product whatsoever. Other populations (Salt Creek, Lost River, and Mound Spring) assayed displayed apparently normal genotypes: all individuals amplified and the samples did not deviate significantly from Hardy-Weinberg expectations (Stockwell et al., in preparation). Thus the null allele appears to occur only in the Malpais Spring population.

The redesigned primer WSP11LO-A, external to the original primer, permitted amplification of all individuals from the Malpais Spring sample (Figure 1). For the three additional populations, amplification by the new primer pair left the microsatellite genotypes unchanged with the following exception: the resulting fragments were larger than the original products since they were being amplified using a primer external to one of the original primers (e.g., amplification of the original 177 bp allele, for example, now yielded a product of 202 bp). The nonnull alleles corresponded to 194, 196, 198, 200, and 202 bp with the new primer pair. The null class of alleles, endemic to the Malpais Spring population, included fragments of size 173, 179, 181, 187, and 189 bp. So an entire class of previously undetected alleles, distinct and nonoverlapping in size compared to the nonnull alleles, had been hidden within the single null allele in the original assays (Figure 1).

Sequence data were generated for 38 nucleotides flanking the microsatellite, including the original lower priming site. A total of 15 sequences were analyzed, including null alleles of size 179 ($N = 1$), 181 ($N = 2$), and 187 ($N = 2$), as well as nonnull alleles of size 194 ($N = 4$), 200 ($N = 4$), and 202 ($N = 2$). All nonnull alleles were identical in flanking sequence to one another and to the original sequence cloned from the *C. tularosa* library (Figure 2). The five sequenced null alleles were also identical to one another and differed from the cloned sequence by a 4 bp deletion within one of the original priming sites. Thus this deletion was the cause of the original null condition. The null alleles also differed consistently from the nonnull alleles by a single G to A transition at a nucleotide position 1 bp removed from the start of the microsatellite array (Figure 2).

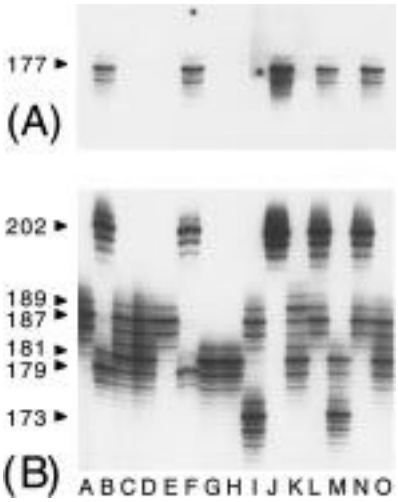


**Figure 1.** Autoradiographs of sequencing gels resolving the amplified products from 15 pupfish from the Malpais Spring population. **(A)** Amplification using the original WSP11 primers (WSP11UP and WSP11LO). **(B)** Amplification of the same templates using the alternative primers (WSP11UP and WSP11LO-A). Note in **(B)** that the alternative primers revealed a host of different alleles that had appeared as a null in the original assays **(A)**. The allele 177 amplified with the original primers in **(A)** corresponds to the 202 bp fragment produced by the new primers in **(B)**.

## Discussion

Null alleles have been reported in a number of studies employing microsatellite loci. In our case the null allele was readily apparent because it occurred with sufficiently high frequency as to be present in homozygotes. Null alleles usually are more difficult to document. For example, had the null allele been infrequent in our population, it would have occurred primarily in heterozygotes and likely would have remained undetected. Null heterozygotes

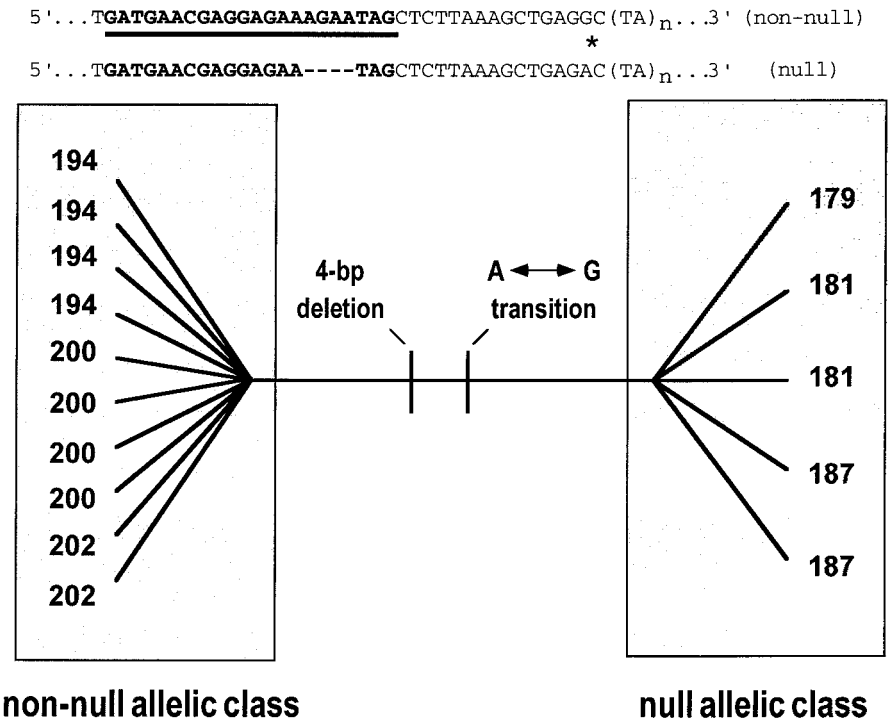


**Figure 2.** (Above) The sequence of the microsatellite WSP11 nonnull allele compared to the sequence retrieved from null alleles amplified from the Malpais Spring population of pupfish. The original primer WSP11LO is underlined. The second AGAA motif is shown as the site of the deletion (relative to the nonnull allele), but in reality any 4 bp segment of the AGAAAGAA sequence could have been the cause of the lesion. The asterisk denotes a point mutation that was perfectly linked to the 4 bp deletion. (Below) A graphical representation of the mutational distinctions in the 38 bp flanking sequence among the total of 15 haplotypes sequenced from the null and nonnull allelic classes. Numbers indicate the sizes (in bp) of the alleles sequenced.

would appear as nonnull homozygotes, and the blank gel profiles in the few true null homozygotes might have been attributed to sample degradation or failed PCR. Furthermore, rare null alleles would be unlikely to cause statistically significant deviations from Hardy-Weinberg equilibrium with the sample sizes employed in most population genetic surveys. Given these detection difficulties, null alleles probably are even more commonplace than indicated by reports in the literature.

In our case the high frequency of the pupfish null allele offered the advantage that null alleles often occurred in homozygous form such that direct sequencing of alternative alleles could be accomplished without the added difficulty of physically isolating haplotypes from heterozygous diploid tissues (e.g., Ortí et al. 1997). Although the microsatellite haplotypes in each such homozygote are not necessarily identical by descent, and hence might in principle retain some sequence variety whose phase (*cis* or *trans*) would remain unspecified, such complications did not arise in our assays. Thus sequences for three different size classes of null alleles (as well as three size classes of nonnull alleles) were obtained without ambiguity.

Few studies have examined the molecular basis of null alleles at microsatellite loci. Three studies conducted previously have documented point mutations that disrupt the problematic priming site (Ishibashi et al. 1996; Lehmann et al. 1996; Paetkau and Strobeck 1995). Two other studies identified short deletions in the priming site with similar effects: Callen et al. (1993) found an 8 bp deletion of one *GGTG* and one of the *TCTG* motifs from the sequence CCTC *TCTG GGTG TCTG* TGTC, and Ede and Crawford (1995) found the lesion to be a 12 bp deletion of a TAAGTTGCGTCC sequence that was preceded directly by an almost perfect tandem repeat, TCAGTTGCGTCC.

In our study a deletion was also found, in this case involving one of a pair of tandemly repeated AGAA sequence motifs (Figure 2) (of course, the evolutionary event could have been an insertion that converted an ancestral null allele into a non-null as assayed by the original primers). Interestingly, in both this study and that by Ede and Crawford (1995), the indels producing null alleles involved repetitive DNA. Though it is difficult to generalize from these few observations, it seems reasonable that tandem repeats should be avoided as PCR priming sites when possible.

In this study the original null allele actually concealed a class of five different microsatellite alleles distinct in size from the various nonnull alleles. This suggests that the 4 bp indel responsible for the null allele arose long enough ago to have accumulated a number of microsatellite length mutations. This suspicion is supported further by the nucleotide substitution that also cleanly distinguished all of the null alleles from the nonnulls.

Another conceivable way that the null alleles could have acquired microsatellite variation would be through recombination with the nonnull alleles. If this were the case the null alleles might be expected to mirror the variation of the original nonnull alleles, but to be shifted downward in length by 4 bp. This scenario is not consistent with our observations: The nonnull alleles were of size 194, 196, 198, 200, and 202 bp, whereas the null alleles were 173, 179, 181, 187, and 189 bp in length.

The two prior reports of null-producing deletions at microsatellite loci (Callen et al. 1993; Ede and Crawford 1995) entailed a situation similar to the present in which null alleles represented distinct size classes relative to the nonnulls. Conversely, for microsatellite nulls caused by point mutations, the allelic size variants appeared to be similar to those of the nonnull alleles (Lehmann et al. 1996; Paetkau and Strobeck 1995).

From the Department of Genetics, University of Georgia, Athens, GA 30602 (Jones, Walker, and Avise) and the Savannah River Ecology Laboratory, Aiken, South Carolina (Stockwell). C. A. Stockwell is currently at the Department of Zoology, North Dakota State University, Fargo, North Dakota. This work was supported by an NIH training grant to A.G.J. and D.W., by an NSF grant and University of Georgia funds to J.C.A., by contract DE-FC09–96SR18546 between the U.S. Department of Energy and the University of Georgia's Savannah River Ecology Laboratory, and by Department of Defense Legacy Funds from USAF, Holloman Air Force Base, New Mexico, to C.A.S. Mike Goodisman, Hsiu-Ping Liu, Margaret Mulvey, Bill Nelson, Hildy Reiser, Guillermo Ortí, Devon Pearse, Kurt Wollenberg, and Lorenzo Zane offered valuable comments on the manuscript. Fish were collected under New Mexico state permit 2887 issued to C.A.S. Thanks to Hildy Reiser and John Pittenger for assistance in collecting fish. Address correspondence to Dr. Avise at the address above.



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